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## **Essential roles of Raf/extracellular signal-regulated kinase/mitogen-activated protein kinase pathway, YY1, and Ca<sup>2+</sup> influx in growth arrest of human vascular smooth muscle cells by bilirubin**

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**Abstract:** The biological effects of bilirubin, still poorly understood, are concentration-dependent ranging from cell protection to toxicity. Here we present data that at high nontoxic physiological concentrations, bilirubin inhibits growth of proliferating human coronary artery smooth muscle cells by three events. It impairs the activation of Raf/ERK/MAPK pathway and the cellular Raf and cyclin D1 content that results in retinoblastoma protein hypophosphorylation on amino acids S608 and S780. These events impede the release of YY1 to the nuclei and its availability to regulate the expression of genes and to support cellular proliferation. Moreover, altered calcium influx and calpain II protease activation leads to proteolytical degradation of transcription factor YY1. We conclude that in the serum-stimulated human vascular smooth muscle primary cell cultures, bilirubin favors growth arrest, and we propose that this activity is regulated by its interaction with the Raf/ERK/MAPK pathway, effect on cyclin D1 and Raf content, altered retinoblastoma protein profile of hypophosphorylation, calcium influx, and YY1 proteolysis. We propose that these activities together culminate in diminished 5 S and 45 S ribosomal RNA synthesis and cell growth arrest. The observations provide important mechanistic insight into the molecular mechanisms underlying the transition of human vascular smooth muscle cells from proliferative to contractile phenotype and the role of bilirubin in this transition.

DOI: <https://doi.org/10.1074/jbc.M111.266510>

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ZORA URL: <https://doi.org/10.5167/uzh-67476>

Journal Article

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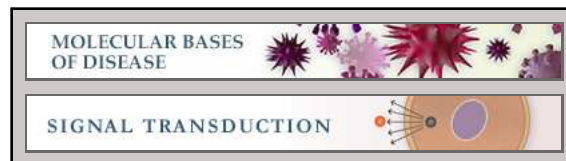
Originally published at:

Stoeckius, M; Erat, A; Fujikawa, T; Hiromura, M; Koulova, A; Otterbein, L; Bianchi, C; Tobiasch, E; Dagon, Y; Sellke, F W; Usheva, A (2012). Essential roles of Raf/extracellular signal-regulated kinase/mitogen-activated protein kinase pathway, YY1, and Ca<sup>2+</sup> influx in growth arrest of human vascular smooth muscle cells by bilirubin. *Journal of Biological Chemistry*, 287(19):15418-15426.

DOI: <https://doi.org/10.1074/jbc.M111.266510>

**Molecular Bases of Disease:**  
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*J. Biol. Chem.* published online January 18, 2012



Access the most updated version of this article at doi: [10.1074/jbc.M111.266510](https://doi.org/10.1074/jbc.M111.266510)

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**Essential roles of Raf/ERK/MAPK pathway, YY1, and Ca<sup>+2</sup> influx in the growth arrest of human vascular smooth muscle cells by bilirubin.**

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Running title: *Human vascular smooth muscle cells response to bilirubin*

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**Keywords:** human coronary artery smooth muscle cells; cellular growth arrest; bilirubin; transcription factor YY1; retinoblastoma protein phosphorylation; Ca influx; calpain II protease; Raf/ERK/MAP pathway.

**Background:** Bilirubin circulates throughout the human cardiovascular system. Its interaction with the vascular wall is not well known.

**Results:** Bilirubin alters Raf/ERK/MAPK pathway, cellular YY1 location and calcium-dependent YY1 proteolysis in human vascular cells.

**Conclusion:** At high physiological levels bilirubin, inhibits cell growth, proliferation, and does not cause apoptosis.

**Significance:** The observations provide opportunities for prevention and treatment of cardiovascular diseases.

## SUMMARY

The biological effects of bilirubin, still poorly understood, are concentration-dependent ranging from cell protection to toxicity. Here we present data that at high nontoxic physiological concentrations bilirubin inhibits growth of proliferating human coronary artery smooth muscle cells by three events. It impairs the activation of Raf/ERK/MAPK pathway and the cellular Raf and cyclin D1 content that results in retinoblastoma protein hypophosphorylation on amino acids Ser608 and Ser780. These events impede the release of YY1 to the nuclei and its availability to regulate the expression of genes and to support cellular proliferation. Moreover, altered calcium influx and calpain II protease activation leads to proteolytical degradation of transcription factor YY1. We conclude that in the serum stimulated human vascular smooth muscle primary cell cultures (hVSMC) bilirubin favors growth arrest and we propose that this activity is regulated by its interaction with the Raf/ERK/MAPK pathway, effect on cyclin D1 and Raf content, altered Rb profile of hypophosphorylation, calcium influx, and YY1 proteolysis. We propose that these activities together culminate in diminished 5S and 45S ribosomal RNA synthesis and cell growth arrest. The observations provide important mechanistic insight into the molecular mechanisms underlying the transition of human vascular smooth muscle cells from proliferative to contractile phenotype and the role of bilirubin in this transition.

## INTRODUCTION

Early studies established that mitochondria might be a major target of bilirubin, leading to uncoupling of oxidative phosphorylation (1). Other studies indicate that bilirubin could inhibit DNA and protein synthesis in some tissues as well as cell lanes, and induce apoptosis (2, 3).

Apart from its toxic effect at high concentrations, bilirubin also seems to play an important role in protecting cells from oxidative damage by acting as a scavenger of peroxyl-, hydroperoxyl- and hydroxyl radicals (4). The extended system of conjugated bilirubin double bonds and the presence of a reactive hydrogen atom underlie its functions as a powerful biological chain-breaking antioxidant supporting the idea of a beneficial role (4). Indeed, recent clinical data associates the increased total serum bilirubin level with lessened susceptibility to peripheral arterial disease (PAD) (5) and coronary artery disease (CAD) (6). Moreover, there is also accumulating evidence from epidemiological studies showing that individuals with high normal plasma bilirubin levels, including individuals with Gilbert syndrome, have a lesser incidence of CAD and carotid vascular plaque formation (7). Thus, there is considerable clinical evidence that elevated serum bilirubin level is associated with a diminished susceptibility to atherosclerotic vascular disease. Studies in rats demonstrate a salutary bilirubin effect through its suppression of vascular neointimal formation in the carotid artery due to impaired activation of MAPKs. Involvements of transcription factor YY1 and the retinoblastoma protein (Rb) in rodents have also been proposed. However, an exact molecular mechanism of bilirubin action on human VSMC remains to be established.

Thus we set out to investigate the mechanisms underlying bilirubin action in primary cell cultures of human coronary artery smooth muscle cells. By treating growth stimulated primary cell culture with physiologically relevant concentrations of bilirubin in a complex with FBS we were able to detect an effect of bilirubin on the Raf/MEK/ERK pathway. We report for specific effect on cyclin D1 content and the Rb profile of

amino acids phosphorylation. Altered influx of calcium, resulting in calpain II protease circuit activation occurs as well. This culminates in protein cleavage of the transcription factor YY1 by calpain II. Furthermore, we observed alterations in the YY1 regulated expression of 5S ribosomal RNA (5S rRNA) and 45S rRNA. Together with the proteolytically lowered YY1 levels, altered Raf/MEK/ERK pathway, cyclin D1 content, and Rb profile of hypophosphorylation, we suggest that this could explain the growth arrest effect of bilirubin on hVSMC. These observations provide new insights into the molecular mechanisms and pathways underlying the switch of hVSMC from proliferative to contractile phenotype and the role of bilirubin in this transition. These novel observations may provide opportunities for prevention and treatment of cardiovascular diseases by laying the ground for the development of novel therapeutic strategies that target these pathways.

## EXPERIMENTAL PROCEDURES

*Cell culture, treatment, immunofluorescence.* hCASM C from different donors, a female Caucasian 46 years, a male Caucasian 21 years and a male Caucasian 49 years, from Cambrex, USA, were cultivated in SmGM-2 (Cambrex, USA) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. All experiments were performed at passages 5 to 8 of the cells under continuous subconfluent conditions. For bilirubin treatment cells were cultivated up to 70% confluence. Cells were first starved for 12 hours in medium with 0.5% fetal bovine serum (FBS) and for another 24 hours in the absence of FBS. After starvation, cells were cultivated in medium with 10% FBS and with or without 100 µM bilirubin. The bilirubin stock solution was prepared in 20 µM NaOH and 10 mg/ml BSA. Cells were harvested for RNA and protein after 0 hours (starved cells), 8, 16 and 24 hours upon cultivation with or without bilirubin. In some experiments 0.5mM EGTA-AM (Molecular Probes) or 1mM EGTA were added to the cell medium 20 min prior harvesting. The intracellular Ca content was compared by treating the cells with 100 nM of fura red for 20 min as recommended by the supplier (Molecular Probes). Fluorescent microscopy (Zeiss Axiovert 135 fluorescence microscope) images of fura red fluorescence at

514 nm excitation, 550-nm emission were collected and the staining intensity was measured in pixels with ImageJ software (NIH, USA) and Adobe PhotoShop. Cell culture without bilirubin served as a negative control. Measurements were performed in triplicates. Cells were prepared for immunofluorescent microscopy as previously described (12).

*Isolation of RNA and RT-qPCR.* Total RNA is isolated from the cells with the RNeasy Mini kit from (QIAGEN) as recommended by the manufacturer. For RT-qPCR experiments, 600 ng total RNA was reverse transcribed using a cDNA Synthesis kit (Invitrogen) with random-oligo primers. For cellular qRT-PCR, 500 ng total RNA was reverse transcribed using the same conditions as above; 1/10 dilutions were used in triplicates with 0.2 µM gene specific primers and 5 µl LightCycler 480 SYBR Green I Master kit (Stratagene) in 20 µl reactions. Gene specific primers were selected (PRIMER BLAST, NIH), synthesized and gel purified. The sequences of the primers are: 5SRNA- CCT CCA GTG GTT GTC GAC TT (forward) and GAA CGA CAC ACC ACC GTT C (reverse); 45SRNA- CCT CCA GTG GTT GTC GAC TT (forward) and GAA CGA CAC ACC ACC GTT C (reverse); β-actin: AAC TGG AAC GGT GAA GGT GAC AGC (forward), TGG CTT TTA GGA TGG CAA GGG ACT (reverse); α-smooth muscle actin: AAT GAG ATG GCC ACT GCC GC (forward), CAG AGT ATT TGC GCT CCG GA (reverse). The relative level of gene expression was normalized to the β-actin gene.

*<sup>3</sup>H-thymidine incorporation, apoptosis, and cell cycle analyses.* For thymidine incorporation hCASM C were cultivated in 24-well plates. 1 µCi of [methyl-<sup>3</sup>H] thymidine (Perkin Elmer) was added per well to measure *de novo* DNA synthesis by determining level of cellular [<sup>3</sup>H] thymidine incorporation.

The results of the individual treatments were obtained in duplicates using a scintillation counter (Beckmann). FITC-labeled annexin V (PromoKine) was used to identify early apoptotic cells following the protocol of the supplier. The number of the annexin V positive cells was counted by fluorescence microscopy in four independent experiments. Cell cycle analysis was performed as previously reported (8). DNA



content analysis of samples was performed in duplicates using a FACS scan (Becton Dickinson) and analyzed with Cellquest software (Becton Dickinson).

**Protein isolation and western blots.** Total cell lysates were prepared by resuspending the cells in RIPA buffer (Boston Bioproducts) supplemented with 1mM EDTA. Nuclear and cytosolic extracts were prepared with the NE-PER kit (Thermo Scientific) following the manufacturer's protocol. The protein concentration of the extracts was determined using the DC Protein Assay (Bio-Rad) according to the manufacturer's protocol with a BSA standard curve. Routinely 40 µg of protein per lane was used for western blot with mouse anti-YY1 antibody (Santa Cruz), chicken polyclonal anti-YY1 antibody (Cell Code, MA), anti-Sp1 antibody (Santa Cruz), the antibodies to total Rb, hypophosphorylated Rb (D20), phospho Rb (Ser 608), phospho Rb (Ser 612), pPhospho Rb (Ser 780), ERK (p42/44 MAPK), Phospho ERK (Tyr 202/204), MEK, phospho MEK (Ser 217/221), mTOR, cyclin D1 were purchased from Cell Signaling Tech, Danvers.

The immunoreactive bands were visualized with the Supersignal West Femto Kit (Pierce). The scanned x-ray films were then analyzed with ImageJ software (NIH, USA) and Adobe PhotoShop.

**Data analysis and statistical methods.** The comparative CT method (Applied Biosystem) was used to analyze the data resulting from the RT-qPCR experiments. Student's *t* test for unpaired results was performed to evaluate differences between two groups. Differences were considered to be significant for values of  $p < 0.05$ . All figures are assembled in FreeHand and Adobe PhotoShop.

## RESULTS

**Bilirubin induces growth arrest in proliferating hVSMC.** Vascular smooth muscle cell proliferation is known to be the key event in vascular response to injury. We examined the impact of bilirubin on cultured primary hVSMC from the coronary artery. Cellular proliferation was measured with the <sup>3</sup>H-thymidine incorporation assay. Cells cultivated with increasing concentrations of bilirubin exhibited lower thymidine incorporation (Figure 1 A, lanes 3-5), in a dose dependent manner, than cells cultivated

without bilirubin (lane 2). The lowest thymidine incorporation was observed in FBS starved cells (lane 1).

To assess if cellular apoptosis contributes to the observed low rate of thymidine incorporation, we performed an annexin V apoptosis assay. The results indicate that the same amounts of cells were undergoing apoptosis when cultivated in the presence or absence of bilirubin (B, lane 1). Tested bilirubin concentrations up to 100 µM did not exhibit a significant increase in apoptosis. Less than 13% of the cells were stained positively for annexin V. In the positive control, significantly more cells were positively stained for annexin V in response to the apoptosis-inducing treatment with TNFα and IL-1β.

To further investigate the mechanism of the underlying bilirubin-induced inhibitory effect on cell proliferation, cell cycle analysis was performed with propidium iodine (PI) DNA staining and subsequent FACS analysis (C). The assay illustrated that the majority (>70%) of the cells that were cultured for 24 hours in complete medium, FBS, and 100 µM bilirubin were growth arrested at the G0/G1 phase of the cell cycle. In the absence of bilirubin, however, significantly fewer cells (<60 %) were found in the G0/G1 phase. In serum starved cells, over 80% of the cells were found in the G0/G1 compartment of the cell cycle. Thus, the sole mechanism by which bilirubin inhibits hVSMC proliferation is likely to be through growth arrest in the G0/G1 phase of the cell cycle. More importantly, the anti-proliferative effect is not due to cell death.

### **Bilirubin alters Rb phosphorylation pattern, cellular YY1 distribution and binding with Rb.**

It is known that cell cycle transition from G0/G1 to S phase is controlled by Rb phosphorylation (9). At least 16 different Rb amino acids are known to be differentially phosphorylated, that leads to altered Rb-protein transcription factors interactions and regulatory functions in gene transcription (10, 11). Therefore, we investigated if bilirubin would change the amino acids phosphorylation profile of the cellular Rb. We performed Western blot experiments of total cell lysates from bilirubin treated and untreated cells, with antibodies that recognize individual phospho-Rb variants (Fig. 2). We observed hypophosphorylation of Rb at SER780 (panel A)

and SER608 in cells cultivated in the presence of FBS and 100  $\mu$ M bilirubin compared to cells cultivated in sole FBS-supplemented medium. These changes are consistent throughout 8, 16 and 24 hours of bilirubin treatment. The hypophosphorylation pattern for SER608 and SER780 resembles the serum-starved cells (lane 1). In contrary, the level of the phospho-SER612 variant does not change significantly in response to bilirubin. Hence the variant serves as an internal control for equal protein loading.

The hypophosphorylated Rb binds YY1 and holds the protein in the cytosol (12). It is not known if the Rb hypophosphorylation on SER608 and SER780 in bilirubin treated cells will be sufficient to bind and prevent the YY1 migration to the nuclei. We verified the Rb-YY1 binding and cellular localization by immunofluorescence and antibodies to Rb (S612) and YY1. Following treatment with FBS together with bilirubin (panel B) most of the cells stained positively for the YY1-Rb complex in the cytosol (orange). In contrast, YY1 stained green in the nuclei of cells that received only FBS. In our second assay for the YY1-hypophosphorylated Rb binding in bilirubin treated VSMC we tested the ability of antibody to YY1 to capture the hypophosphorylated Rb (panel C). Consistent with the immunofluorescence results (panel B), YY1-specific antibody captured ~35% of the hypophosphorylated Rb (lane 2) in the cytosolic extract from 16 hours bilirubin treated cells (lane 1). Pre-immune mouse antibody served as a negative control (lane 3).

Our experiments argue that treatment with 100  $\mu$ M bilirubin in serum stimulated hVSMC leads to hypophosphorylation of Rb at SER608 and SER780. The phosphorylation level at position Ser612 however, remains high and is seemingly independent of the treatment. Importantly, the hypophosphorylated Rb variant in the bilirubin treated cells is active to bind YY1 impeding the release to the nuclei.

**Bilirubin has an effect on the Raf/ERK/MAPK pathway.** The Raf/MEK/ERK kinase pathway is a major pathway that has been shown to influence Rb phosphorylation as well as cell cycle transitions. We assayed if this pathway is influenced by hVSMCs treatment with bilirubin. Western blot experiments were performed with cell lysates from bilirubin treated and untreated

cells using antibodies that are specific to various total- and phospho-forms of the kinases Raf, MEK-1/2, and ERK-1/2 proteins (Fig. 3). We observed that, while total protein levels were constant, MEK-1/2, and ERK-1/2 were less phosphorylated 8 hours after bilirubin treatment when compared to bilirubin untreated cells (Fig. 3, lanes 2-3). The inhibitory effect of bilirubin on phosphorylation was most pronounced in ERK 1/2 throughout the tested 24 hours (Fig. 3, lanes 1-7). Bilirubin also showed an inhibitory effect on the cellular cyclin D1 level through the tested 24 hours. In addition, less total Raf protein was detectable in the 24 hours bilirubin fraction (lanes 6). The protein level of  $\beta$ -actin seems not to be altered by bilirubin treatment remaining nearly similar to the levels in the growth-arrested control cells before serum stimulation (Fig. 3, lanes 2-7). This protein serves as a control for equal protein loading.

The data argue for a specific inhibitory effect of bilirubin on Raf (Ser338), MEK (Ser217/221), and ERK (Thr202/Tyr204) phosphorylation. In addition, bilirubin treated hVSMC have significantly less cyclin D1 protein, as well as Raf. The observed changes coincide with the bilirubin-specific profile of Rb hypophosphorylation (10) (Fig. 2) and cellular growth arrest (Fig. 1).

**Bilirubin exposure increases  $\text{Ca}^{2+}$ -influx and activation of calpain II that manifests in proteolytical YY1 cleavage.** Previously we reported that YY1 is a direct Rb target in VSMC (12). Hypophosphorylated Rb is known to restrain YY1 in the cytosol, preventing its migration into the nucleus to regulate genes that are directly involved in the hVSMC transition from growth arrest to the S phase (12). In western blot experiments we compared the nuclear YY1 content in nuclear extracts from cell cultures after serum stimulation for 8 hours and 24 hours in the presence or absence of bilirubin (Fig. 4, A). The results consistently revealed presence of >2 times less immunologically reactive full length YY1 in bilirubin treated cells (lanes 3 and 5) when compared to the serum stimulated control cells (lanes 2 and 4).

The low YY1 protein content could reflect an altered YY1mRNA content in response to bilirubin treatment. Accordingly, we measured the

YY1 mRNA content by RT-qPCR in bilirubin treated and untreated cells. The YY1 mRNA content was found to remain relatively constant (panel B). Thus, the bilirubin-related alteration in YY1 protein content is likely to be post-translationally regulated. To assay for degradation of the protein upon bilirubin treatment we performed Western blots with polyclonal anti-YY1 antibodies (panel c) and total cell extract. The blots indicate the presence of faster migrating YY1 ~35 kDa fragments (13) in the bilirubin cell culture lysates suggesting for an intracellular proteolysis of YY1 (C, lane 1). The cleavage is YY1-specific since two other assayed Zn-finger transcription factors, Sp1 and mTOR, remain intact (C, lane 5-8). The proteolytical YY1 cleavage is likely to be caused by the activation of the  $\text{Ca}^{2+}$ -dependent protease calpain II, that cleaves YY1 in other cell types (13). We carried out 8 hours bilirubin treatment in the presence of the potent membrane permeable calcium chelator EGTA-AM and EGTA that does not enter the cells. We observed that YY1 cleavage was diminished when the EGTA-AM was added to the medium (C, lane 3). The extracellular  $\text{Ca}^{2+}$  chelator EGTA, however, has little effect on reducing the suggested calpain II-YY1 cleavage (C, lane 4) (23).

Changes in the intracellular  $\text{Ca}^{2+}$  content in response to bilirubin could activate calpain II and potentially account for the observed YY1 cleavage. We measured and compared the intracellular  $\text{Ca}^{2+}$  level in serum-stimulated cells that are cultivated in the presence of 100  $\mu\text{M}$  bilirubin (D) for 1, and 8 hours. The data clearly demonstrates that 1 hours treatment with bilirubin results in more than 2 times  $\text{Ca}^{2+}$  level elevation when compared to the control cells. The  $\text{Ca}^{2+}$  level remains high 8 hours after adding bilirubin to the cell culture medium.

Collectively, our data document the ability of bilirubin to alter the  $\text{Ca}^{2+}$  influx and possibly induce the activation of the  $\text{Ca}^{2+}$ -calpain II circuit in hCASM. We suggest that these cellular events ultimately manifest in the cleavage of YY1.

**Altered expression of RNA polymerase I, polymerase II, and polymerase III transcribed genes in response to bilirubin.** We observed alterations in cell growth, the Raf/ERK/MAP kinase pathway, and the pattern of Rb

phosphorylation, together with the reduced cellular YY1 content in bilirubin-treated cells. This could result in altered transcription of genes with cell cycle transition functions that are known to be regulated by YY1 and Rb-YY1 interaction. YY1 participates in the regulation of all three DNA dependent RNA polymerases, RNA polymerase I (pol I), polymerase II (pol II), and polymerase III (pol III) (18-21). It is also known that ribosome biogenesis is tightly regulated during the cell cycle and differentiation processes [25]. We thus tested if cells treated with bilirubin exhibit an altered transcript levels of 45S rRNA and ribosomal 5S rRNA (Fig. 5) by RT-qPCR analysis. The 45S rRNA that is transcribed by pol I does not decrease within the initial 8 hours of treatment. However, a decrease is observed 16 and 24 hours after adding bilirubin to the growth medium. The pol III transcribed 5S rRNA significantly decreased earlier after the first 8 hours. Importantly, bilirubin treatment coincides with a time-dependent elevation in mRNA content of the pol II transcribed VSMC differentiation marker, the smooth muscle alpha actin.

This suggests that bilirubin could arrest hVSMC cell growth by inhibiting the expression of pol I and pol III transcribed 5S rRNA and 45S rRNA. While the rRNAs content goes down, bilirubin has a positive effect on the mRNA content of the pol II transcribed marker of VSMC differentiation, the smooth muscle alpha actin, which is known to be repressed by YY1 (12).

## DISCUSSION

There is considerable clinical evidence that elevated serum bilirubin levels are associated with a diminished susceptibility to atherosclerotic vascular disease (5-7). However, these findings do not necessarily implicate bilirubin as the protective agent at the cellular level. Since vascular smooth muscle cell proliferation is a key event in the vascular response to injury and that bilirubin is known to inhibit the development of atherosclerotic vascular disease, we examined the impact of bilirubin on hVSMC proliferation in primary human cell cultures. Treating the cells with bilirubin inhibited the cell proliferation in a dose dependent manner, while extensive proliferation was found in the control cells (no bilirubin). In addition, while FBS-stimulated



control cells began to progress throughout the cell cycle upon serum starvation, as seen by FACS analysis, the bilirubin treatment retained the cells in the G0/G1 arrest. More importantly, the hVSMC response to bilirubin at the tested high physiological concentration does not cause apoptosis as shown by annexin V staining. This absence of apoptosis is significant given recent reports that apoptosis of VSMCs can trigger the development of neointima formation (24).

Our data suggests that at certain concentrations bilirubin triggers a non toxic cascade of molecular events that block hVSMC proliferation. Bilirubin clearly alters the phosphorylation status of Rb, a protein with crucial G1/S checkpoint functions that are essential for the cell cycle transitions (9). The bilirubin treatment coincides with specific dephosphorylation of Rb at Ser780 and Ser608 while preserving the phospho Ser612. Such profile of Rb phosphorylation is likely to be essential for keeping the hVSMC in a growth arrested state. Importantly, it facilitates Rb interactions with specific transcription factors, including YY1 (12) and its cytoplasmic retention with direct consequences on gene transcription (12).

One of the major cell signaling pathways known to alter the phosphorylation status of Rb at Ser780 and Ser608 is the Raf/ ERK/ MAPK pathway (16). We observed that a significant portion of ERK-1/2 is hypophosphorylated in bilirubin treated cells. Similarly, Raf and MEK are also hypophosphorylated, albeit to the lesser extent. It remains unclear how bilirubin promotes the accumulation of the non-phosphorylated protein forms. Bilirubin could, for example, inhibit Raf phosphorylation by inhibiting pathways upstream of Ras in a SMC specific manner (25). It is known that Ras directly interacts with and activates Raf by phosphorylation. Raf next phosphorylates and activates MEK, which in turn phosphorylates and activates ERKs. Therefore, the observed low cellular Raf protein content and hypophosphorylation in response to bilirubin could trigger low levels of MEK and ERKs phosphorylation and pathway inactivation. Through Raf, bilirubin could also act in a cascade-independent fashion, for example by inactivating transcription factor NF- $\kappa$ B. Such scenario could explain, at least in part, the lower level of apoptosis in bilirubin treated hVSMC. In airway smooth muscle cells, Taillé and colleagues

suggested that bilirubin could also modulate the phosphorylation of ERK by a redox mechanism (17). Furthermore, bilirubin could also modulate other cell signaling pathways that are cross-talking with the ERK/MAPK pathway. The p38 pathway, for instance, could interact with the ERK pathway to alter phosphorylation of up- and downstream proteins. Such a hypothesis is supported by our previous observation that bilirubin treatment coincides with accumulation of underphosphorylated p38, a major kinase in the MAPK signaling pathway in mouse and rat SMCs (8).

The observation that the cellular cyclin D1 protein content is highly reduced in response to bilirubin treatment is significant. Cyclin D1 is one of the major cellular cyclins in terms of its functional importance for the cell cycle transitions. Cyclin D-Cdk4/6 complex partially phosphorylates Rb to trigger S phase progression (26). The observed low level of cyclin D1 together with the accumulation of underphosphorylated ERK could explain the appearance of hypophosphorylated Rb and the growth arrest in the bilirubin- treated cell cultures.

The effect of bilirubin on cell signaling pathways is most likely a complex event including redox- and other mechanisms that control the cell cycle status. The exact mechanism that control the low cellular Raf and cyclin D1 levels in response to bilirubin, however is important and remains to be determined.

Bilirubin treatment coincides with a reduced YY1 protein level. In this study we did not witness changes in YY1 mRNA levels. The lower YY1 protein content is a result of a bilirubin-triggered specific YY1 proteolytic cleavage. No proteolysis of other Zn-finger transcription factors including Sp1 and mTOR could be observed. The increase in YY1 specific cleavage is likely to be a consequence of activated calcium influx and calpain II protease activation. Supporting evidence corroborating the idea that bilirubin could have an effect on the calcium influx, on calpain II activation in hVSMC, and YY1 degradation, was previously reported for neuronal cell cultures (22). Cleavage of YY1 by calpain II is likely to play important regulatory functions in the myogenic differentiation (13). The calcium influx activation together with the involvement of YY1 cleavage mechanism in the hVSMC growth arrest-response

to bilirubin is novel. Targeting proteases to a transcriptional machinery may represent a unique feature in the vascular gene regulation and the response to injury.

Bilirubin significantly inhibited the expression of the pol I and pol III transcribed 45S rRNA and 5S rRNAs. The availability of these RNAs ultimately determines the ribosomal assembly and the cellular potential for growth and proliferation (18, 19). As YY1 and Rb are both implicated in the regulation of pol I and pol III transcribed genes (20, 21) changes in the status of Rb phosphorylation together with the decreased protein level of YY1 most likely underlies the reduced 45S and 5S rRNA level of expression. It is possible that the bilirubin-related Rb profile of amino acids hypophosphorylation retains YY1 in the cytosol, forming Rb-YY1 complex that prevents YY1 migration to the nuclei and subsequent activation of 5S and 45S rRNA transcription. Such a notion is directly supported by our previous data showing that a direct correlation exists between levels of cellular hypophosphorylated Rb, cytosolic Rb-YY1 content, and the hCASM cell cycle phase (12). In addition, proteolytic cleavage also contributes to the functional nuclear YY1 deficiency, as shown in this study.

Taken together, our data suggest that bilirubin

treatment inhibits hVSMC growth through two mechanisms. Firstly, the impaired activation of Raf/ERK/MAPKs together with the reduced content of cellular cyclin D1 and Raf proteins results in inhibited Rb phosphorylation on serines 608 and 780. These events impede the release of transcription factors that are important for VSMC growth, such as YY1. Secondly, there is an increase in specific YY1 protein cleavage as a result of possible bilirubin-related change in the calcium influx and calpain II protease activation. The reduced cellular protein level of the transcription factor YY1 itself results in a suppressed capacity to induce ribosomal RNA synthesis and cell proliferation.

Our observations suggest that at high-normal levels bilirubin does not cause human VSMC cell death. As we have shown in this study, there are candidate molecules and pathways that could explain the association of high-normal bilirubin levels and the maintenance of human vascular homeostasis. These observations provide new mechanistic insights into the molecular players and pathways underlying the transition of hVSMC from proliferative to contractile phenotype and the response to bilirubin. They may provide opportunities for the development of novel therapeutic strategies to target vascular response to injury and neointimal formations.

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**Sources of Funding:** The work was supported by the National Heart, Lung, and Blood Institute (R01HL062458 to AU, R01HL46716, R01HL69024 to FWS), a fellowship from the Swiss Public Health Institute to AE. **11**(8):558-72

## Figure Legends

**Figure 1. Effect of bilirubin treatment on hVSMC proliferation and cell cycle transition.** Serum starved hVSMCs were cultivated in medium containing different concentrations of bilirubin and without bilirubin. A) [ $^3$ H]-thymidine incorporation was measured for serum starved cells (lane 1), FBS stimulated cells (lane 2), and cells that are cultivated in medium with FBS and increasing concentrations of bilirubin, 10  $\mu$ M (lane 3), 50  $\mu$ M (lane 4) and 100  $\mu$ M (lane 5) as indicated below the bars. The [ $^3$ H] content in cells that are cultivated in medium with FBS without bilirubin was used as reference (100%). B) Annexin V apoptosis assay. Cells were cultivated with 100 $\mu$ M bilirubin (bilirubin), without bilirubin (control), or with the proapoptotic mixture of TNF $\alpha$  (400 U) and IL-1 $\beta$  (100 U) as indicated below the bars. The number of apoptotic cells that stain positively for FITC-annexin V was determined by fluorescence microscopy. The total number of cells was taken as reference (100%). C) Bilirubin treatment resulted in cellular growth arrest at the G0/G1 phase of the cell cycle as illustrated by the FACS profile. The cell cycle position of serum starved cultures, and cultivated cells with bilirubin and without bilirubin, as indicated above the profiles, was determined by PI staining of DNA and subsequent FACS analysis. Less than 1 % of apoptotic cells were present in the cell cultures independently of the cell culture conditions. Each experiment is also illustrated by diagrams below the profiles where the bars present the quantity of cells in G0/G1, S, and G2 phases. Results from the experiments were obtained in triplicates. Three independent experiments produced consistent results. Values are mean SEM (n=3) in each group.

**Figure 2. Bilirubin alters the Rb phosphorylation profile, YY1-Rb binding, and cellular YY1 localization.** Serum starved hVSMCs were cultivated in medium with 10% FBS and 100  $\mu$ M bilirubin and compared for Rb phosphorylation profile and presence of YY1-Rb complexes to cells that were cultivated without bilirubin. A) Total cell lysates (100  $\mu$ g protein) were analyzed for content of phosphorylated Rb at Ser780, Ser6012, and Ser608 by Western blot with specific antibodies as indicated above the blots: lane 1- Ser608 and Ser780 content in serum deprived cells; lane 2- Ser608 and Ser780 content after 8 hours treatment with FBS and bilirubin (+); lane 3- 8 hours treatment with FBS alone (-); lane 4- 16 hours treatment with bilirubin; lane 5- 16 hours treatment with FBS alone; lane 6-24 hours treatment with bilirubin(+); lane 7- 24 hours treatment minus bilirubin(-). The duration of treatment with FBS and bilirubin (+) and minus bilirubin (-) is shown above the lanes. The western blot results with anti-Rb (S612) antibody are presented as follows: lanes 1, 2, 3, 4 – lysates from cells that are FBS stimulated cells at different time points after stimulation as indicated above the lanes; lanes 5, 6, 7, 8- FBS stimulated cells together with bilirubin at different time points after stimulation. The intensity of the specific bands is measured in pixels as shown below the western blot panels. The bilirubin (100  $\mu$ M) presence (+) gray or absence (-) black bar is shown at the right. Values are mean SEM of 3 independent experiments ( $P < 0.05$  vs. FCS-stimulated control). B) Indirect immunofluorescent staining of cells with rabbit anti- Rb (S612) (red) antibody and mouse anti-YY1 antibodies (green). Cells received FBS alone and together with bilirubin as indicated above the panels (original magnification x200, inserts original magnification x400). FITC-labeled anti-mouse and Alexa595-labeled anti rabbit antibodies were used for visualization by fluorescent microscopy. The co-localization of both proteins results in orange staining. C) Cytosol extract (0.3mg) from 16 hours bilirubin-treated cells were mixed and incubated with an affinity matrix containing covalently attached monoclonal antibody to YY1. Captured proteins were analyzed by Western blot with Rb-specific antibody. Samples are as follows: input lysate (lane 1, IN); bound and eluted by boiling in 1%SDS (lane 2, B); captured with pre-immune mouse antibody (lane 3, C). The apparent molecular mass (indicated to the *right* of the *panel*) of Rb was estimated by comparing the migration to protein size markers. The migration of the hyperphosphorylated (p) and the hypophosphorylated (h) Rb is shown on the left.

**Figure 3. Phosphorylation and total protein levels of Raf/ERK/MAPK pathway members in response to bilirubin.** Western blot analysis of total cell lysates from serum starved hVSMCs (lane 1); cells treated with(+) and without (-) bilirubin for 8 hours (lanes 2 and 3); 16 hours (lanes 4 and 5); 24 hours (lanes 6 and 7). Protein specific antibodies were used to monitor changes in protein content: total  $\beta$ -actin protein content was used as a standard for equal protein loading; Raf protein content; phospho Raf; total MEK 1/2; phospho MEK 1/2; ERK 1/2; phospho-ERK 1/2; total cyclin D1. The identity of the specific antibodies is shown at the top of the panels. 40  $\mu$ g of protein is loaded in each of the lanes. Each experiment is also illustrated through diagrams below the panels in which the bars present the quantity (pixels) of immunologically reactive protein. The molecular mass of the proteins is shown at the left of the panels. The bilirubin (100  $\mu$ M) presence (+) or absence (-) in the cell cultures is shown above the lanes together with the duration (h) of treatment. Results are reproducible within 3 independent experiments. The bars present the average value of 3 independent experiments ( $P < 0.05$  vs. FCS-stimulated control).

**Figure 4.  $\text{Ca}^{2+}$  influx and proteolytic cleavage of YY1 in response to bilirubin treatment.** Bilirubin (100 $\mu$ M) was added to the culture medium (+) together with FBS to stimulate growth. 8h and 24 h later cells were harvested for mRNA and protein analyses as indicated above the lanes and below the bars. Experiments with FBS alone without bilirubin were used as a control (-). A) Total RNA and cDNA were prepared for RT-PCR. RT-PCR reactions were conducted with YY1 gene-specific primers as indicated below the bars. Reactions with  $\beta$ -actin primers were used as an internal control. The bars represent fold changes in the presence of bilirubin ( $P < 0.04$  vs. FCS-stimulated cells). B) The cellular YY1, Sp1, and mTOR protein content is monitored in total cell lysates by Western blot with protein-specific antibody as

indicated on the right of the panels. All reactions received equal amounts of protein (50µg). Lane 1, total cell lysate from cells after 24 hours treatment with bilirubin; lane 2, cells in the absence of bilirubin; lane 3, cell lysate from cells that are grown in the presence of bilirubin and EGTA-AM (AM); lanes 4, cells are grown in the presence of bilirubin and EGTA; lane 5 and 6, Sp1-specific antibody identifies the presence of Sp1 protein in the lysates; lane 7 and 8, mTOR- specific antibody identifies the presence of total mTOR protein. The YY1, Sp1, and mTOR antibodies reactive bands are indicated on the right. The size markers migration is shown on the left (kDa). The results are consistent between 2 independent experiments. C) Cells are serum stimulated without (-) and with (+) bilirubin (100µM). One hour and 8 hours later cells are treated with the  $\text{Ca}^{2+}$  indicator fura red for 20 min and the intracellular  $\text{Ca}^{2+}$  content is shown by fluorescent microscopy at 550 nm excitation. The red color corresponds to the intracellular fura red -  $\text{Ca}^{2+}$  complex. The red color intensity in the cells is measured in pixels. Measurements are performed in triplicates. Three independent experiments produced consistent results ( $P < 0.03$ ). A fluorescent microscopy picture of bilirubin treated (+) and control (-) cells (8 hours) is shown bellow the bars.

**Figure 5. Effect of bilirubin on pol I, pol II, and pol III transcribed RNAs in hVSMC.** Total RNA was isolated from bilirubin treated and untreated cell cultures and used in RT-qPCR reactions with gene-specific primers for: pol II transcribed smooth muscle actin; 45S rRNA transcribed by pol I; 5S rRNA transcribed by pol III as indicated above the diagrams. The bars represents the fold change in specific RNA content using the  $\beta$ -actin mRNA level as an endogenous reference gene that does not change in response to treatment. PCR reactions were conducted with total RNAs isolated from cell cultures after 0, 8, 16, and 24 hours of bilirubin treatment as indicated below the bars. Three independent experiments produced consistent results (S.D.  $\sim \pm 3\%$ ).

**Figure 6. Model of the molecular mechanism of bilirubin induced growth arrest in hVSMC.** Bilirubin treatment decreases cellular protein content of cyclin D1 and Raf. It also inhibits phosphorylation and activation of members of the Raf/ERK/MAPK pathway, resulting in the accumulation of hypophosphorylated at Ser608 and Ser780 Rb that is a key suppressor of cell cycle transition from G0/G1 into S phase. The hypophosphorylation profile of Rb facilitates Rb-YY1 binding, retention in the cytosol and consequent nuclear YY1 depletion. Bilirubin also activates intra cellular  $\text{Ca}^{2+}$  influx,  $\text{Ca}^{2+}$ -calpain protease circuit activation, and YY1 cleavage, further altering the nuclear YY1 availability for transcription. The reduced level of nuclear YY1 results in altered mRNA content of pol I, pol II, and pol III transcribed genes with important functions in the VSMC cell cycle control.



**Figure 1**

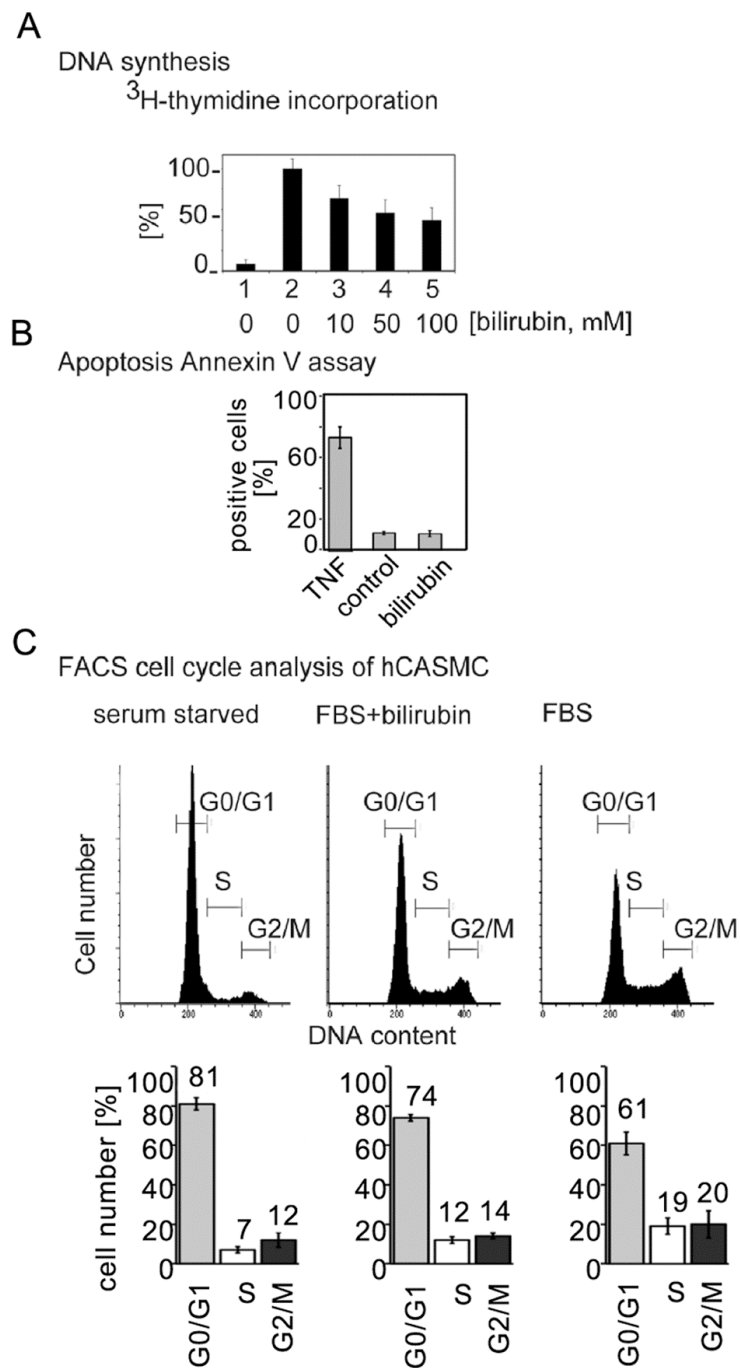
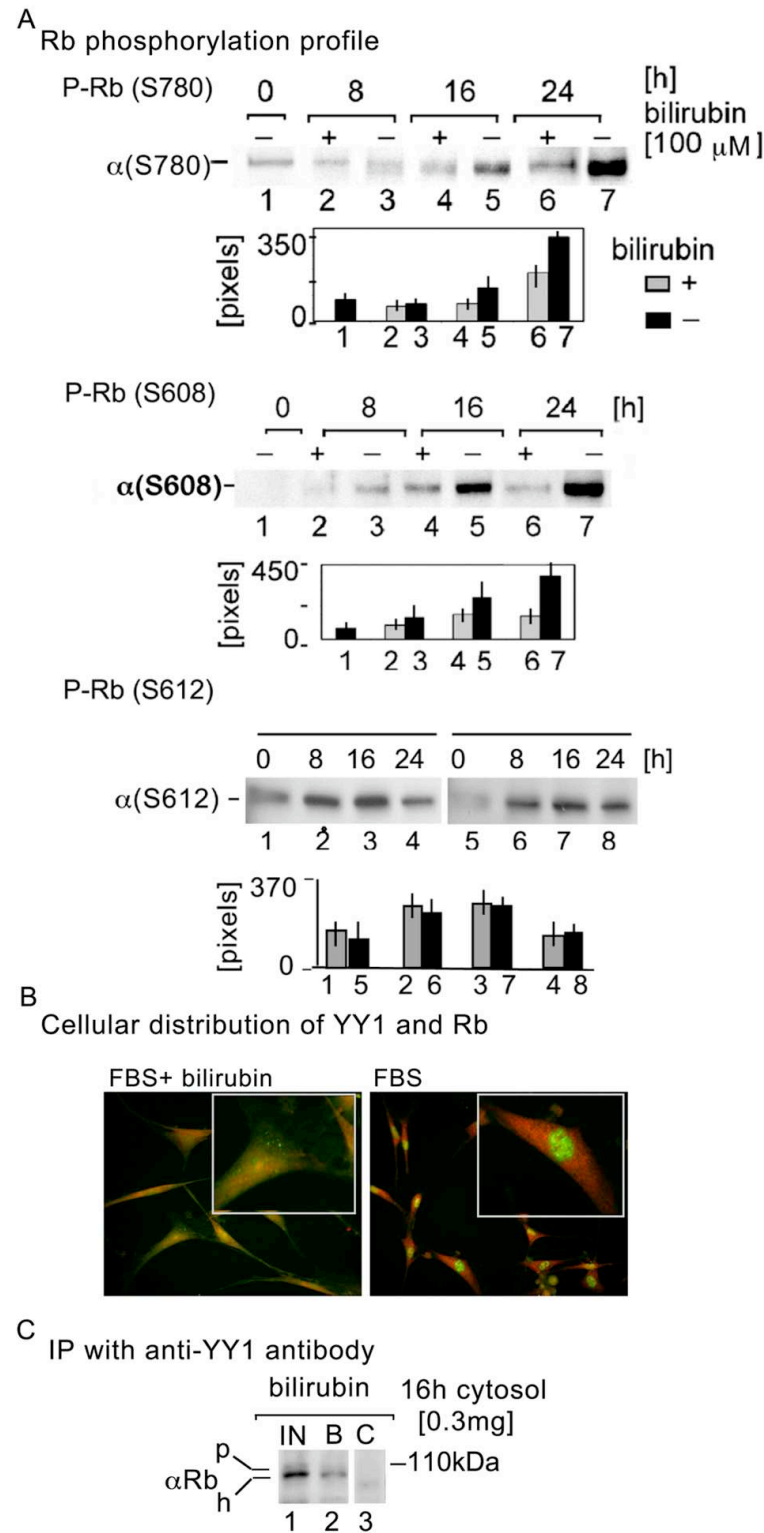
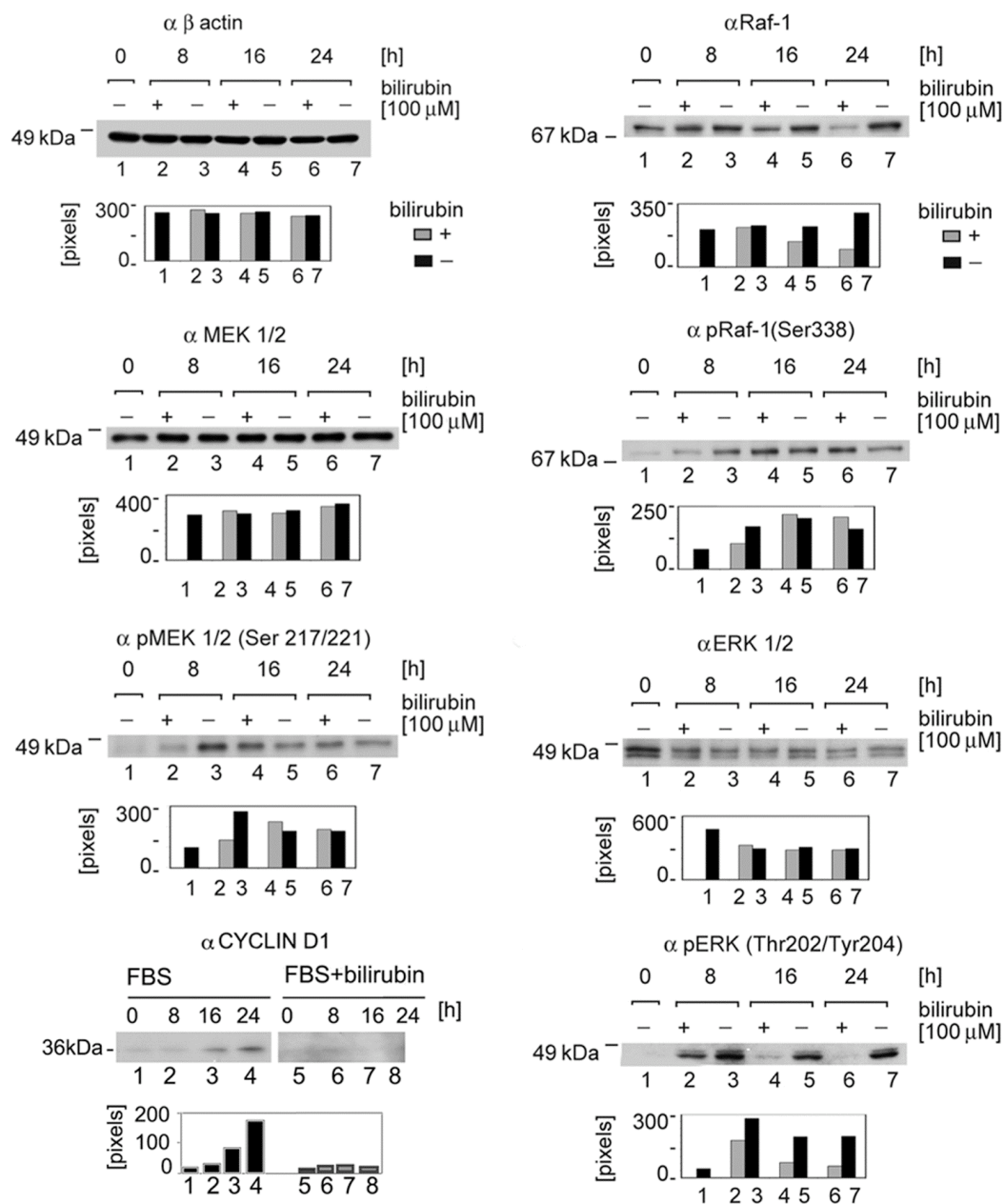


Figure 2



**Figure 3**

**Figure 4**

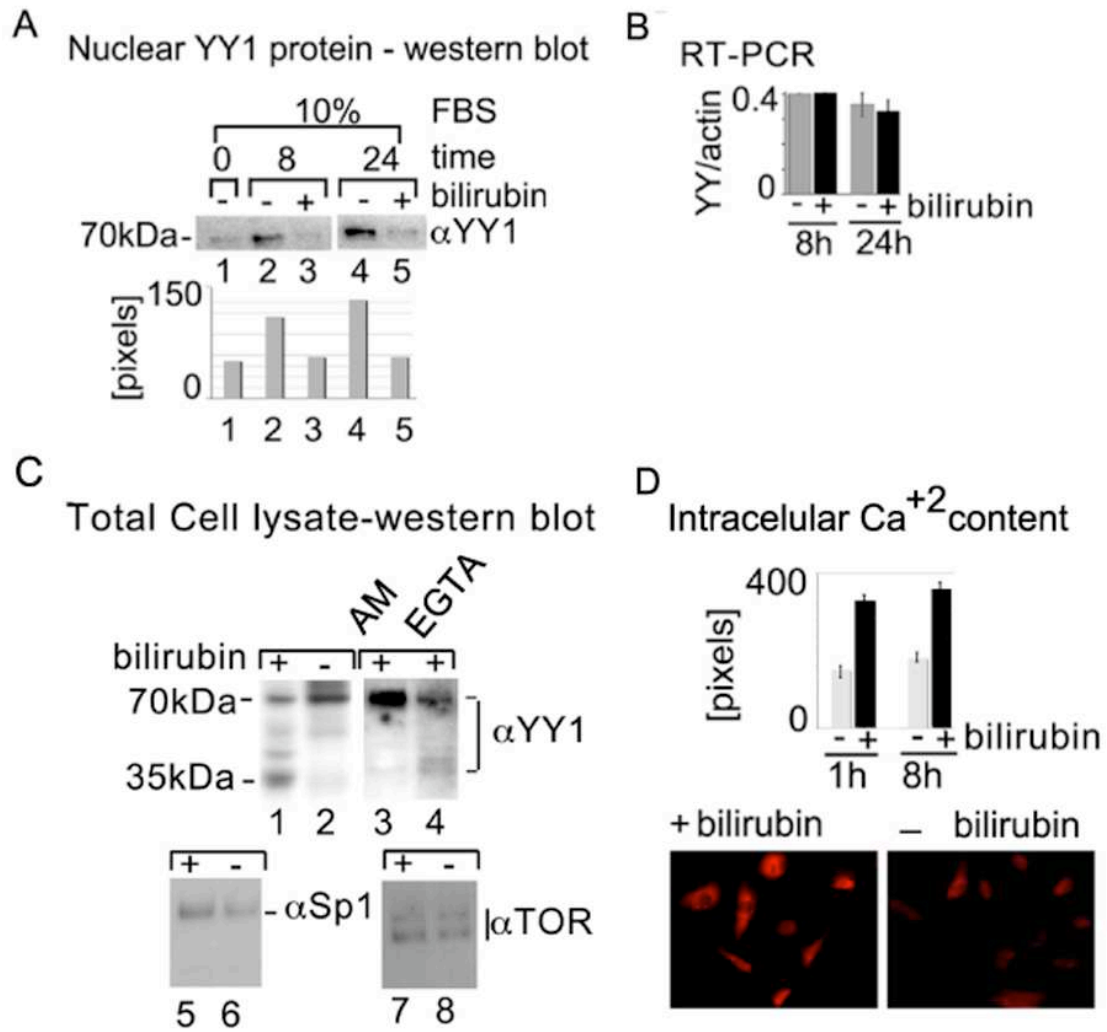


Figure 5

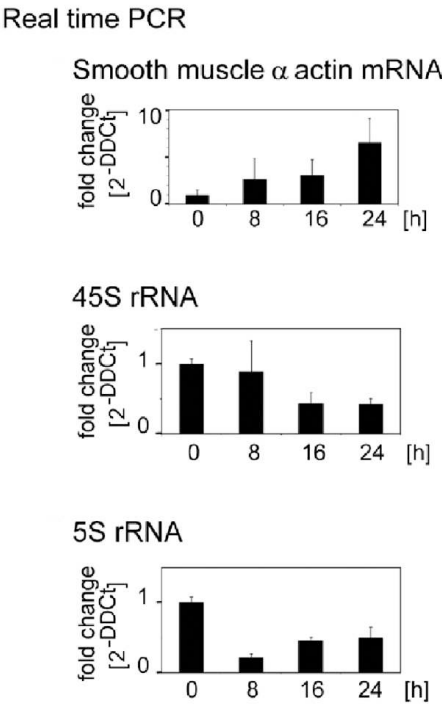


Figure 6

